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Award Number: DAMD17-00-1-0537

TITLE: Preclinical Evaluation of Gene Therapy for NF2 Lesions in Mouse Models Using Amplicon Vectors and Prodrug Activation

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REPORT DATE: November 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20020416 132

**REPORT DOCUMENTATION PAGE***Form Approved*  
OMB No. 074-0188

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<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> November 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Oct 00 - 1 Oct 01)	
<b>4. TITLE AND SUBTITLE</b> Preclinical Evaluation of Gene Therapy for NF2 Lesions in Mouse Models Using Amplicon Vectors and Prodrug Activation			<b>5. FUNDING NUMBERS</b> DAMD17-00-1-0537	
<b>6. AUTHOR(S)</b> Xandra O. Breakefield, Ph.D., Vijaya Ramesh, Ph.D., S.M. Kumar, Y. Tang, M. Giovannini, R. Weissleder				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Massachusetts General Hospital Boston, Massachusetts 02114  E-Mail: <a href="mailto:breakefield@helix.mgh.harvard.edu">breakefield@helix.mgh.harvard.edu</a>			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> Report contains color				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> Spontaneous schwannomas were detected in a transgenic murine model of NF2 by Magnetic Resonance Imaging (MRI). Tumors were detected in the central and peripheral nervous system, in smooth muscles of the uterus and limbs, and in the intercostals muscles of adult mice. These tumors display isointensity with other organs following T1 weighted image sequences and hyperintensity following T2-weighted image sequences, which are characteristic features of schwannomas in humans. Hematoxylin and Eosin (H and E) staining and immunohistochemistry indicate that these tumors consist of schwannomas and Schwann cell hyperplasias. Tumors stain positively for S-100, a marker for cells of Schwann cell origin, and VSV-G, a marker for the mutated NF2 transgene placed under the control of the Schwann cell-specific PO promoter. Preliminary evidence suggests that these tumors are highly infectable with the replicational-conditional virus HSV vector, hrR3, which contains the reporter gene, lacZ. In addition, we have been successful in producing meningiomas in the ventricles and brain parenchyma of immunodeficient mice by injection of a malignant human meningioma cell line, F5, into the ventricles. This cell line also appears to highly infectable with HSV vectors. Thus, two NF2 tumor models have been established in mice appropriate for testing of therapeutic HSV vectors.				
<b>14. SUBJECT TERMS</b> schwannomas, NF2, MRI, hrR3, Schwann cell				<b>15. NUMBER OF PAGES</b> 34
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

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Annual Report : Nov.1, 2001

Title: "Preclinical evaluation of gene therapy for NF2 lesions in mouse models using amplicon vectors"

Investigators: Drs. Shanta M. Kumar, Vijaya Ramesh, & Xandra O. Breakefield,  
Massachusetts General Hospital, Boston, MA

## **Introduction:**

Neurofibromatosis type 2 (NF2) is an inherited condition in which patients have a tendency to develop multiple schwannomas and meningiomas (Eldridge, 1981). In this condition, germ-line mutations in the NF2 gene are associated with major structural impairment of the encoded protein, merlin (Trafletti et al., 1993). Biallelic inactivation of the NF2 gene occurs in both sporadic and familial schwannomas and meningiomas, suggesting that NF2 is a tumor suppressor gene (Bianchi et al., 1994; Bijlsma et al., 1994; Jacoby et al., 1996; Sainz et al., 1994; Twist et al., 1994). In this study, we have used an animal model for NF2 developed by Dr. Marco Giovannini which employs transgenic mice that express in Schwann cells a mutant NF2 protein similar to that found in NF2 patients (Giovannini et al., 1999). This mutation consists of an interstitial deletion in the amino-terminal domain of NF2 and the transgenic mice demonstrate a high incidence of Schwann cell-derived tumors and Schwann cell hyperplasia. We have detected schwannomas in these mice using MRI in collaboration with Dr. Ralph Weissleder's group. We are currently investigating how infectable these schwannomas are with HSV vectors and whether injection of a replication-conditional HSV vector, hrR3, into tumors arrests their growth. In order to model meningiomas found in NF2 patients, human meningioma cell lines have been characterized for merlin expression and infectability with HSV vectors, and used to generate brain tumors in immunodeficient mice.

## **Body:**

### **1) Detection of schwannomas in NF2 transgenic mice by MRI**

We received a shipment of 13 transgenic founders (10 males and 3 females), ages 13-20 months, from Dr. Marco Giovannini in France. These animals typically demonstrate Schwann cell hyperplasias and/or schwannomas in 80 % of transgenic mice by 9 months of age, especially in the spinal ganglia and around peripheral nerve endings in skeletal muscle. These transgenic mice [PO-Sch (39-121)] express mutant NF2 proteins in Schwann cells, similar to a mutant protein found in some human NF2 patients.

The transgenic founders went through a 6 week period of quarantine and pathogen testing and were then cleared for entry into the MGH transgenic facility. Genotyping in the form of tail clipping and PCR analysis was performed on these mice to confirm that they have the mutated NF2 transgene (Fig. 1). We have been establishing a breeding colony by backcrossing these transgenic mice to wild type FVBN mice and then backcrossing the F1 generations with each other. The offspring are being tested for the mutant NF2 gene by tail clipping and PCR analysis. These mutant offspring will be maintained for experiments for the remaining two years of the project.

We have conducted MRI in collaboration with Dr. Yi Tang in Dr. Ralph Weissleder's group at MGH on all of these transgenic mice. Overall, approximately 77 % (10/13) of these mice showed putative tumors by MRI. More specifically, 31 % (4 of 13) of these mice showed tumors in the intercostal muscles and 15 % (2 out of 13) had tumors in the limb muscles. Figure 2 illustrates MRI images of a male mouse which had

tumors in the muscles of the right forelimb (Panels A-C) and in the intercostal muscles (D-F). Imaging sequences included conventional T1 and T2 weighted spin echo sequences with a 512 X 192 matrix, 8 cm field of view, and a 1.5 mm slice thickness, yielding a spatial resolution of 156  $\mu$ m X 312 $\mu$ m X 1500  $\mu$ m. Using a T1W1 sequence (T1W1), the tumors displayed isointensity with other organs (Fig.2A, D). Under the T2W1 fat saturation (FS) sequence, the tumors were hyperintense (B, E). After contrast administration (GDT1), the tumors demonstrated strong enhancement (C, F).

Since the images resulting from the T1 W1, T2FS, and GDT1 sequences showed a signal intensity characteristic of schwannomas (Abe et al., 2000; Hayasaka et al., 1999; Hayashi et al., 1996; Soderlund et al., 1994) and this transgenic strain of mice has a high incidence of schwannomas (Giovannini et al., 1999), we believed this tumor was an NF2 derived schwannoma, and this was confirmed by histology (Fig. 3) and immunohistochemistry (Fig. 4). For histopathological analysis, H and E staining was performed on paraffin embedded sections. The tumor was dispersed among the muscle fibers (Fig. 3A, B) of the right forelimb. Schwann cell hyperplasias were also evident within the muscle fibers (Fig. 3C).

Immunohistochemistry, using a polyclonal antibody to S-100 (Dako Corporation, Carpinteria, CA), on tumor sections from the right forelimb of this mouse also supported the Schwann cell origin of these tumors (Fig. 4). Positive immunostaining was present in paraffin sections from the intramuscular tumor (Fig. 4A), but absent in sections from nontumor tissue, and absent when incubated with secondary antibody alone (Fig. 4C). Schwann cell hyperplasias were also evident in the nerves innervating the limb muscle

(Fig. 4). This data indicates that this intramuscular tumor contains cells of Schwann cell lineage.

## **2) Correlation of MRI imaging and histopathology in NF2 transgenic mice**

Approximately 66 % ( 2 out of 3 mice imaged by MRI) of the female mice had uterine tumors. Figure 5 illustrates MRI images of a uterine tumor one of these mice. Using a T<sub>2</sub>W1 weighted image sequence (T1W1), the tumor displays isointensity with other organs (Fig. 5A). Using the T<sub>2</sub>W1 fat saturation sequence, the tumor has marked hyperintensity (Fig. 5C) and is well-defined with a sharp margin. These imaging characteristics are similar to those observed following the detection of schwannomas in human patients (Abe et al., 2000; Hayasaka et al., 1999; Hayashi et al., 1996; Soderlund et al., 1994)

Those mice which had tumors, as diagnosed by MRI, were sacrificed for histopathological analysis. Necropsy was performed with the help of Dr. Rod Bronson at Harvard Medical School. Tumor tissue was fixed in 10 % formalin prior to being embedded in paraffin, sectioned, and stained with H and E. A necropsy, a gross examination, and histology confirmed that this female mouse had a uterine tumor. Figure 6 illustrates the gross anatomy of this uterine tumor, while Figure 7 shows the histology confirming the MRI diagnosis. The H and E staining of uterine tumor sections reveals that the cells comprising the tumor have relatively abundant, faintly eosinophilic cytoplasm without discernible cell margins (Fig. 7A), and blunt-ended spindle nuclei (Fig. 7B), which are characteristic features of Schwann cells in schwannomas (Woodruff et al., 2000). Histology of other tissue removed from the animal confirmed that the large

intestine had a colonic polyp with some dysplasia, and the gut had a small leiomyoma and a few tumors which resembled schwannomas.

Overall, histopathology demonstrated that schwannomas and Schwann cell hyperplasias were found in 75% (3 of 4) mice of both sexes examined. These data are consistent with studies indicating that these NF2 dominant negative transgenic mice have a high incidence of Schwann cell-derived uterine tumors (Giovannini et al., 1999) and demonstrate that such tumors are detectable in living animals by MRI. However, in one animal, MRI did not detect a tumor, but histology showed a schwannoma in the anterior horn of the spinal cord (Fig. 10A, B, C). In this case, the tumor cells invaded the white matter of the spinal cord and disrupted the normal symmetry of the spinal cord.

### **3) Further characterization of schwannomas in NF2 transgenic mice**

Immunohistochemical studies were performed on sections of tumors from those transgenic mice which had tumors indicated by the MRI and histological analysis. The Schwann cell origin of these tumors was examined by staining with an antibody to S-100, which is a marker for cells of Schwann cell origin. Figure 4 illustrates that the tumor shown in Figures 2 and 3 stains positively for S100. Likewise, the uterine tumor illustrated in Figures 5-7 also stains positively for S-100. These data indicate that the tumors detected by MRI and histology in the NF2 transgenic mice PO-Sch (39-121) have Schwann cell origin, and are thus, schwannomas.

Other immunohistochemical studies confirm that the tumor sections are positive for VSV-G, the marker for the mutated NF2 transgene which is under the control of the Schwann cell-specific PO promoter (Fig. 9). Histological staining for myelin indicates



that the schwannomas do not stain for myelin, consistent with previous evidence of disaggregation of myelin sheaths in vestibular human schwannomas (Sans et al., 1996). Figure 11 illustrates the lack of myelin staining in the schwannoma which invades the anterior horn of the spinal cord illustrated in Figure 10.

We have also investigated the infectability of these schwannomas with the replication- conditional HSV vector, hrR3, which is capable of replication only in dividing cells, thereby killing them. Since most cells in the adult nervous system are non-dividing, this cytotoxicity is selective for tumor cells. hrR3 contains the reporter gene lacZ to facilitate tracking of vector infected cells (Boviatsis et al., 1994). Three days after infection of the tumors with hrR3, the tumors were removed, fixed, sectioned, and analyzed by histology for  $\beta$ -galactosidase expression by staining the sections with X-gal buffer [1 mg/ ml 5-bromo-4-chloro-3-indoxyl  $\beta$ -galactosidase, 5mM  $K_3Fe(CN)_6$ , 5mM  $K_3Fe(CN)_6 \cdot 3H_2O$ , 2mM  $MgCl_2$  in 0.1M sodium phosphate buffer (pH 7.4)] at 37 °C for 12h. Preliminary experiments suggest that the schwannomas are highly infectable with hrR3. Figure 12 illustrates  $\beta$ -galactosidase expression in tumor tissue infected with hrR3 3 days following infection (Panel A), as compared to the lack of  $\beta$ -galactosidase expression in tumor tissue which was not injected with hrR3 (B). In order to determine if the cells infected with hrR3 were schwannomas, we also immunostained tsections of tumors which were infected with hrR3 with a polyclonal antibody to S-100. Figure13 illustrates that  $\beta$ -galactosidase expression and S-100 staining share a similar pattern of distribution in tumor tissue infected with hrR3, suggesting that the schwannoma cells themselves are infectable with hrR3.

Future experiments include investigating whether application of the HSV-derived hrR3 to these tumors reduces their growth. hrR3 has a mutated ribonucleotide reductase gene, so that hrR3 replicates selectively in cells with high levels of endogenous ribonucleotide reductase, such as tumor cells (Yoon et al., 1998). hrR3 also encodes the HSV-thymidine kinase gene, which can convert the pro-drug, ganciclovir, into a metabolite that is toxic to dividing cells. Thus, addition of ganciclovir to hrR3 infected cells may enhance the ability of hrR3 to destroy tumor cells (Boviatsis et al., 1994). We also plan to clone the luciferase gene into hrR3 in order to test infectability of this viral vector in schwannomas *in vivo* using a recently developed bioluminescence imaging system which can detect luciferase expression in living animals available in Dr. Ralph Weissleder's laboratory. Assuming that the schwannomas are infectable *in vivo* with hrR3, future experiments will include investigation of how infection of the schwannomas with hrR3 combined with ganciclovir treatment affects the growth of these tumors, as accessed by volumetric MRI analysis.

## **Development of a meningioma animal model**

### **a) Characterization of the merlin status of meningioma cell lines**

In order to create a meningioma animal model, we screened 13 meningioma cell lines derived from meningiomas of human patients. These meningioma cell lines were tested first for the presence of merlin, the protein encoded by the NF2 tumor suppressor gene by western blotting and immunohistochemistry. Of these cell lines, 8 are positive for immunoreactive merlin, while 5 of these cell lines do not express merlin, by western blot analysis (Fig. 14). For example, the meningioma cell line,

MN-52, does not express merlin, while the malignant meningioma cell line F5 is merlin positive (Fig. 14).

Immunocytochemical studies also demonstrated that this F5 meningioma cell line expresses merlin, using a monoclonal antibody to merlin, 1C4, developed in Dr. Vijaya's Ramesh's laboratory. Further studies showed that this cell line expressed two markers for meningioma cells: vimentin and the epithelial membrane antigen (EMA) (Louis, 2000). Figure 15 illustrates that F5 expresses vimentin (Panel A) and that the immunofluorescence is specific to vimentin, since the controls, preimmune sera (B) and secondary antibody alone (C) do not result in immunofluorescence.

#### **b) Meningioma cell lines are infectable with HSV amplicon vectors**

We have been testing whether human meningioma cell lines, both merlin-positive and merlin-negative, are infectable with the HSV amplicon vectors, HG CX, a standard HSV amplicon, and HSV/EBV/RV, a hybrid amplicon, both of which contain an expression cassette for EGFP. Infectability of the meningioma cell lines with HSV amplicon vectors was assessed by Fluorescence Activated Cell Sorting (FACS) analysis using a fluorescein filter, as a function of Multiplicity of Infection [MOI; transducing units (t.u.) per cell] or by counting the number of GFP positive cells. The meningioma cell line, MN-52, was found to be highly infectable with HG CX, with over 50 % infection at a MOI of 3 t.u. per cell. Likewise, the malignant meningioma cell line F5 is highly infectable with both HSV amplicon vectors, HG CX ), and with the hybrid amplicon HSV/EBV/RV, as indicated by the large number of green cells at MOI= 0.5 to MOI = 2 , 48 hrs after infection (Figure 16).

**c) *In vivo* studies involving injection of meningioma cell lines into the ventricles of nude mice**

We have also conducted *in vivo* studies to determine whether we can identify a human meningioma line which can form tumors in mouse brain. Merlin-positive or merlin-negative meningioma cells ( $5 \times 10^8$  cells) were injected into the right brain ventricles of immunodeficient (nude) mice and tumor growth was monitored in these animals by MRI, animal health, and histopathology. Injection of the merlin-negative MN-52 into the ventricles did not result in tumor formation. However, injection of the merlin-positive meningioma cell line F5 did result in tumor formation, assessed by MRI and histology. Figure 17 illustrates the histopathology demonstrating that a tumor formed in the right ventricles and invaded into the surrounding parenchyma by 4 weeks following injection of F5 into the ventricle.

### **Key Research Accomplishments:**

- Detection of spontaneous schwannomas in a transgenic NF2 murine model for NF2 by MRI
- Correlation of tumor diagnosis by MRI and histopathological analysis
- Confirmation of Schwann cell origin of schwannomas and Schwann cell hyperplasias found in a transgenic murine model for NF2
- Demonstration of infectability of schwannomas *in vivo* with the replication-conditional HSV vector, hrR3
- Characterization of merlin status of 13 human meningioma cell lines
- Demonstration of infectability of meningioma cells in culture with HSV amplicon vectors
- Formation of meningioma tumors in the ventricles of immunodeficient mice with invasion of the brain parenchyma following intraventricular injection of human malignant meningioma cells

### **Reportable Outcomes:**

This work has resulted in a manuscript entitled: "Detection of schwannomas by MRI in a transgenic murine model for NF2", by Kumar, S. M., Tang, Y., Giovannini, M., Weissleder, R., and Breakefield, X. O., in preparation.

## **Conclusions:**

Our findings demonstrate for the first time that MRI can be used to detect schwannomas in a transgenic murine model for human NF2. Histology and immunohistochemistry have confirmed the Schwann cell origin of these tumors and expression of the marker, VSV-G, demonstrates the role of the mutated NF2 transgene in their oncogenesis. The ability to detect these schwannomas in living animals in a murine model with MRI, a noninvasive technique, will allow us to target spontaneous tumors for treatment and to follow their growth and volumetric changes in response to therapeutic interventions. With regard to development of a murine meningioma model, we have shown that a malignant human meningioma cell line can form tumors in the ventricles, and invade the surrounding brain parenchyma following injection into the ventricles of immunodeficient mice. This opens the way to future studies in which gene therapy can be tested in these tumors in a murine model as preclinical assessment of the potential for clinical application.

Importantly, both schwannoma and meningioma tumors in these mouse models have been shown to be highly infectable with HSV vectors, which serve as the platform for therapeutic interventions. One of the vectors tested is a replication-conditional virus bearing the viral thymidine kinase gene which has already been shown to have therapeutic efficacy in mouse models of gliomas (Boviatsis et al., 1994), and have formed the basis of a phase 1 clinical trial in human glioblastoma patients (Markert et al., 2000).

Over the coming year, we will be evaluating the therapeutic efficacy of HSV recombinant hrR3 vectors combined with ganciclovir administration in schwannoma

models by evaluation of tumor volume over time and of tumor-selective virus replication by histochemistry of the lacZ reporter. In addition, we will test a therapeutic HSV amplicon vector in both tumor models. This amplicon vector will bear an apoptotic gene, Interleukin 1 $\beta$ -converting enzyme (ICE) (Yu et al., 1996), under the control of a tetracycline inducible promoter (Freundlieb et al., 1999), with direct injection of vector into the lesion and subsequent “turn-on” of the transgene with a tetracycline analogue, which should result in reduction of tumor size. Amplicon vectors have very low intrinsic toxicity and hence should be readily compatible with potential human trials.

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Appendices: Please see attached figures



Figure 1. For genotyping purposes, PCR was performed on DNA isolated from the tails of the transgenic mice [PO-Sch (39-121)], which have an in-frame interstitial deletion in the amino terminal domain of NF2, representing a natural mutation observed in humans. These NF2 transgenic mice were obtained from Dr. Marco Giovannini and have been shown to spontaneously form schwannomas and Schwann cell hyperplasias (Giovannini et al., 1999). The primers used for this genotyping of the mice were GAP 3.3 and TAGanti. The 320 bp fragment represents the mutated NF2 transgene found in these transgenic mice. No band is detected in wildtype FVBN mice. A 320 bp band is found when PCR is performed with primers and mutated NF2 DNA construct (+), while no band is present (-) with primers alone.

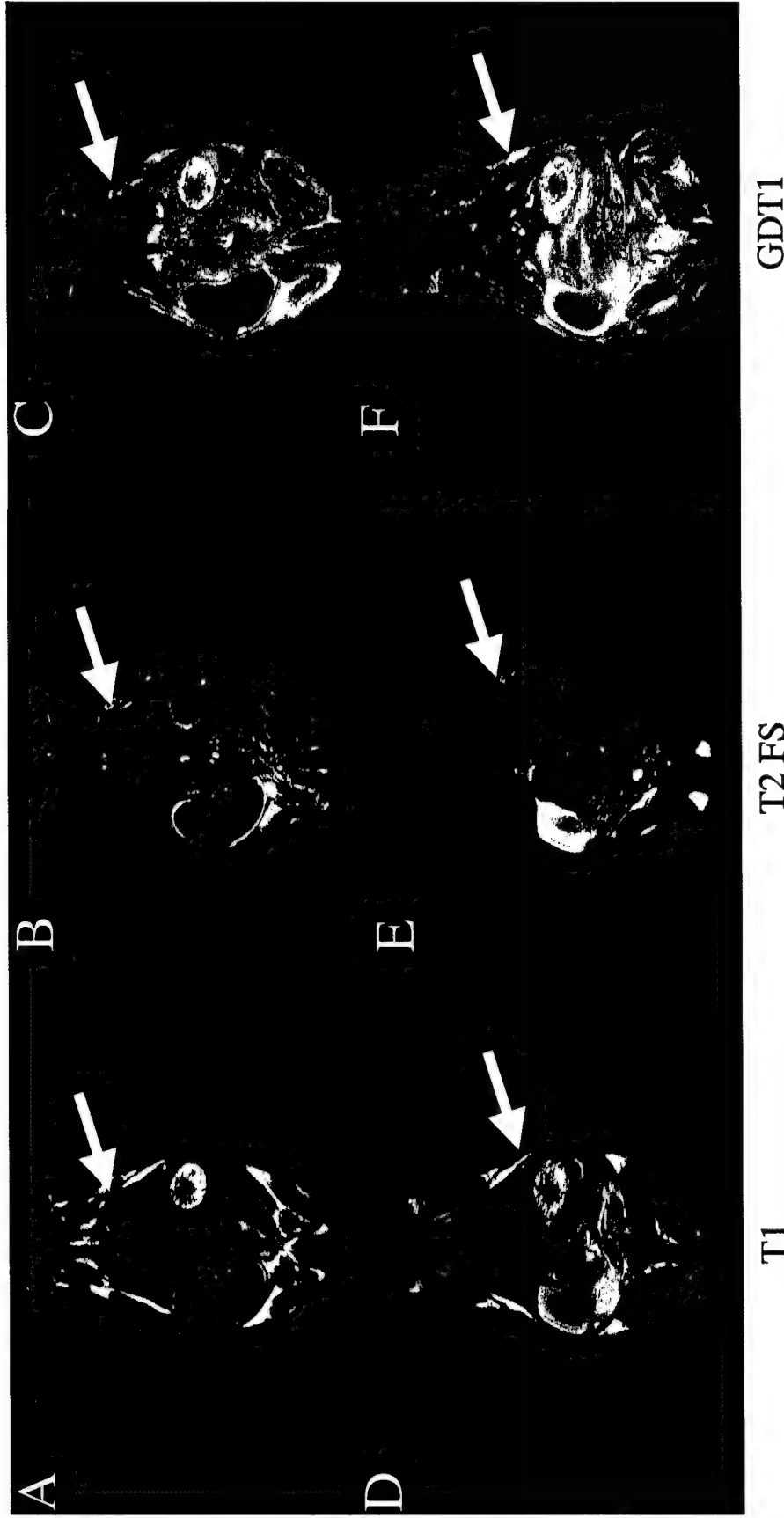


Figure 2. Magnetic resonance images of multiple intramuscular tumors in a NF2 dominant negative transgenic mouse. An anesthetized transgenic male mouse (age 18 months) was taped to a plexiglass plate, which was positioned immediately below a 3-inch surface coil. Imaging sequences included conventional T1 and T2 weighted spin echo sequences with a 512 x 192 matrix, 8 cm field of view, and a 1.5 mm slice thickness, yielding a spatial resolution of 156  $\mu$ m X 312  $\mu$ m X 1500  $\mu$ m. A fat saturation sequence (FS) was also run with the T1 and T2 sequences. T1 and T2-weighted MR images, in the presence of fat saturation and contrast enhancement (Gd-DTPA) are shown. The arrows correspond to a tumor in the right forelimb (A-C) and in the intercostal muscles (D-F). (C, D) Using a TW weighted image sequence (T1WI), the tumor displays isointensity with other organs (A,D). Using the T2WI fat saturation sequence, the tumors have marked hyperintensity (B and E) and are well defined with a sharp margin. The tumor has a homogenous intensity on both T1 and T2WI. After contrast administration (C, F), the tumors showed strong enhancement. T1WI and T2WI images have a signal intensity representative of schwannomas, and this transgenic strain of mice has a high incidence (70 %) of schwannomas (Giovannini, 1999). This is consistent with a NF2-derived schwannoma tumor, which was confirmed by histology and immunohistochemistry (See Figures 3 & 4).



Figure 3. Histopathology of the tumor from the same mouse which was imaged by MRI as shown in Figure 2. Hematoxylin and Eosin (H and E) staining was performed on paraffin embedded sections. The tumor was dispersed among the muscle fibers (A, 10X), (B, 20X) of the right forelimb. Schwann cell hyperplasias are also evident within the muscle fibers (C, 50X).

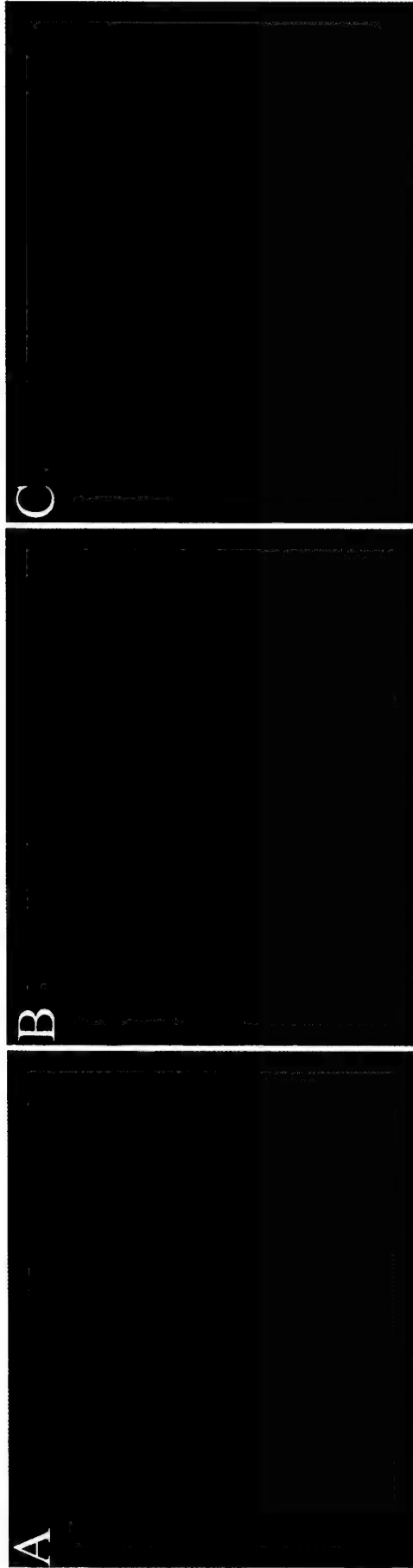


Figure 4. Immunohistochemistry on tumor sections from the right forelimb of the mouse illustrated in Figures 2 and 3 using a monoclonal antibody to S-100, which identifies cells of Schwann cell origin. Positive immunostaining was present in paraffin sections from the intramuscular tumor (A, 40x), but absent in sections from nontumor tissue, and absent when treated with secondary antibody alone (B, 40x). Schwann cell hyperplasias were evident in the nerves innervating the limb muscle (B, 40x). The specific S-100 staining of this intramuscular tumor indicates that the tumor is expressing a protein which is also expressed in Schwann cells and is consistent with Schwann cell origin.

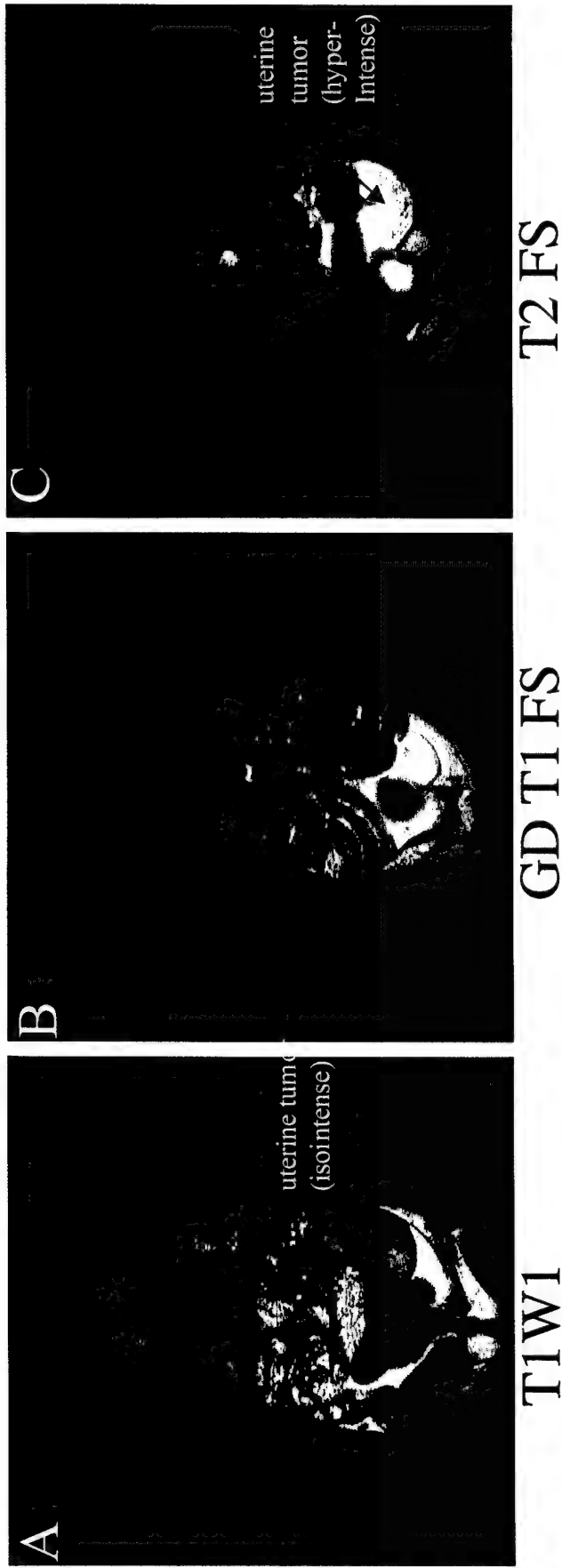
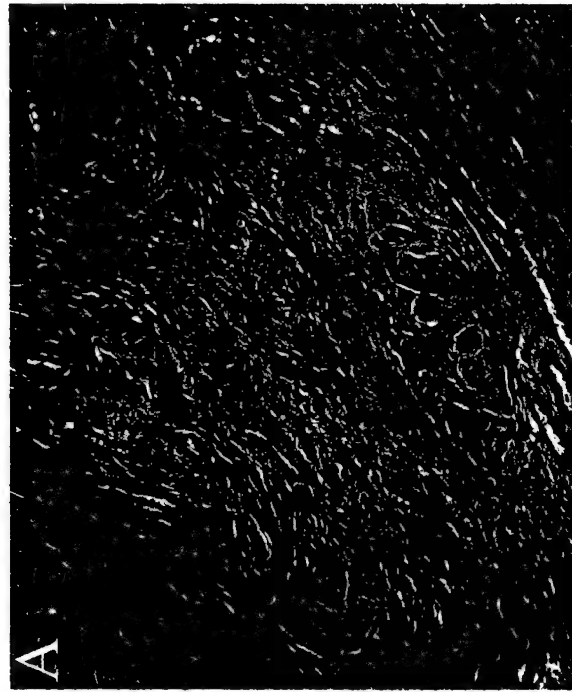


Figure 5. MRI of a uterine tumor in a NF2 dominant negative transgenic mouse [PO-Sch (39-121)]. An anesthetized transgenic female mouse (age 18 months) was taped to a plexiglass plate, which was positioned immediately below a 3-inch surface coil. Imaging sequences included conventional T1 and T2 weighted spin echo sequences with a 512 x 192 matrix, 8 cm field of view, and a 1.5 mm slice thickness, yielding a spatial resolution of 156 X 312 X 1500  $\mu$ m. A fat saturation sequence (FS) was also run with the T1 and T2 sequences. T1 and T2-weighted MR images, in the presence of fat saturation and contrast enhancement (Gd-DTPA) are shown. The arrows correspond to the uterine tumor. Using a TW weighted image sequence(T1 W1), the tumor displays isointensity with other organs (A). Using the T2W1 fat saturation sequence, the tumor has marked hyperintensity (C) and is well-defined with a sharp margin.

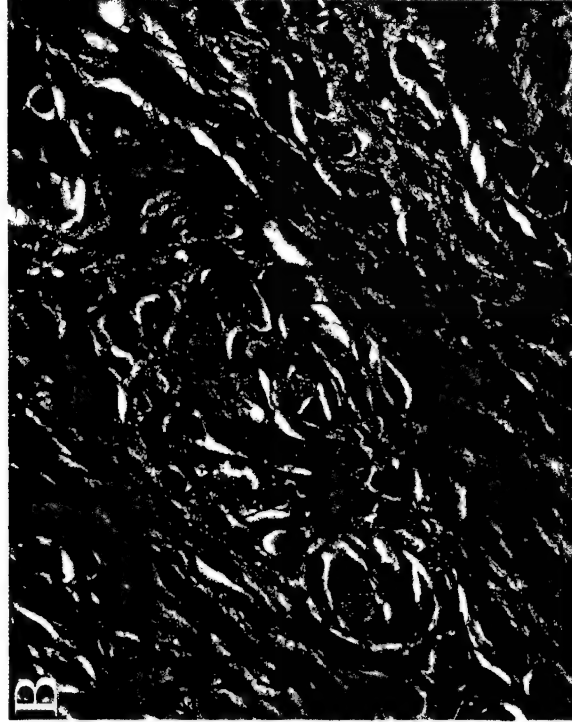


uterine  
tumor

Figure 6. Gross anatomy of female NF2 transgenic mouse which was MRI imaged and illustrated in Figure 5. A necropsy revealed that this mouse had a huge uterine tumor (see black arrow), and putative tumors in the large intestine (see swollen area, blue arrow) and gut, which were all confirmed by histology (Figure 7).



20x



100x

Figure 7. Hematoxylin and Eosin (H and E) staining of the uterine tumor which was depicted in Figures 5 & 6. Note that the cells comprising the tumor have relatively abundant, faintly eosinophilic cytoplasm without discernible cell margins (A, 20x), and blunt-ended spindle nuclei (B, 100x), which are characteristic features of Schwann cells in schwannomas (Woodruff et al., 2000).



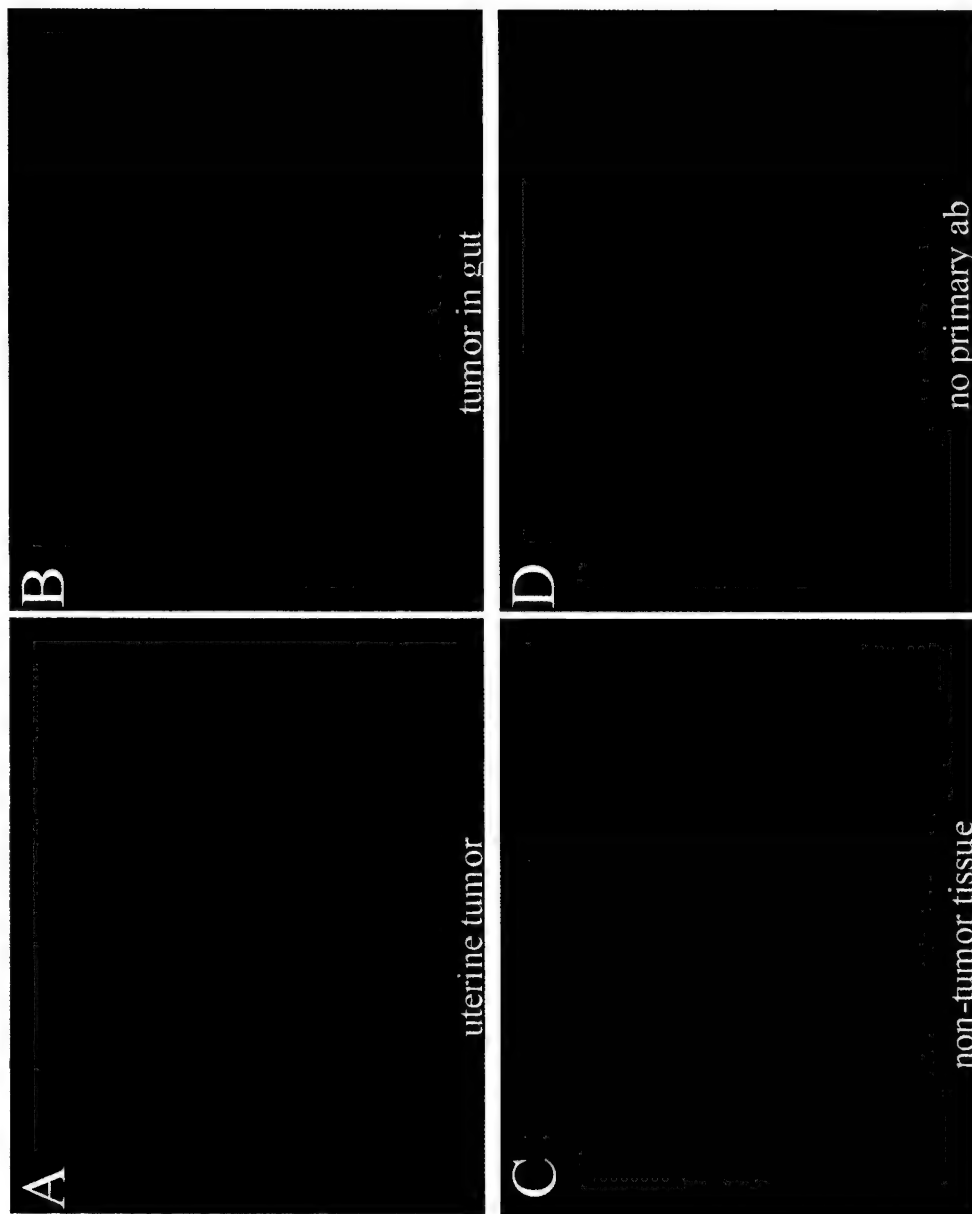


Figure 8. Schwann cell origin of uterine tumor and tumor in gut from mouse depicted in Figure 6. A polyclonal antibody to S100 was used to detect cells of Schwann cell origin on paraffin embedded section. Positive immunostaining was present in the uterine tumor (A, 20X), and the tumor in gut (B, 20X). No immunostaining was present in nontumor tissues (C, 20X), normal uterine sections, or in uterine tumor sections with secondary antibody alone, without primary antibody (D, 20X).

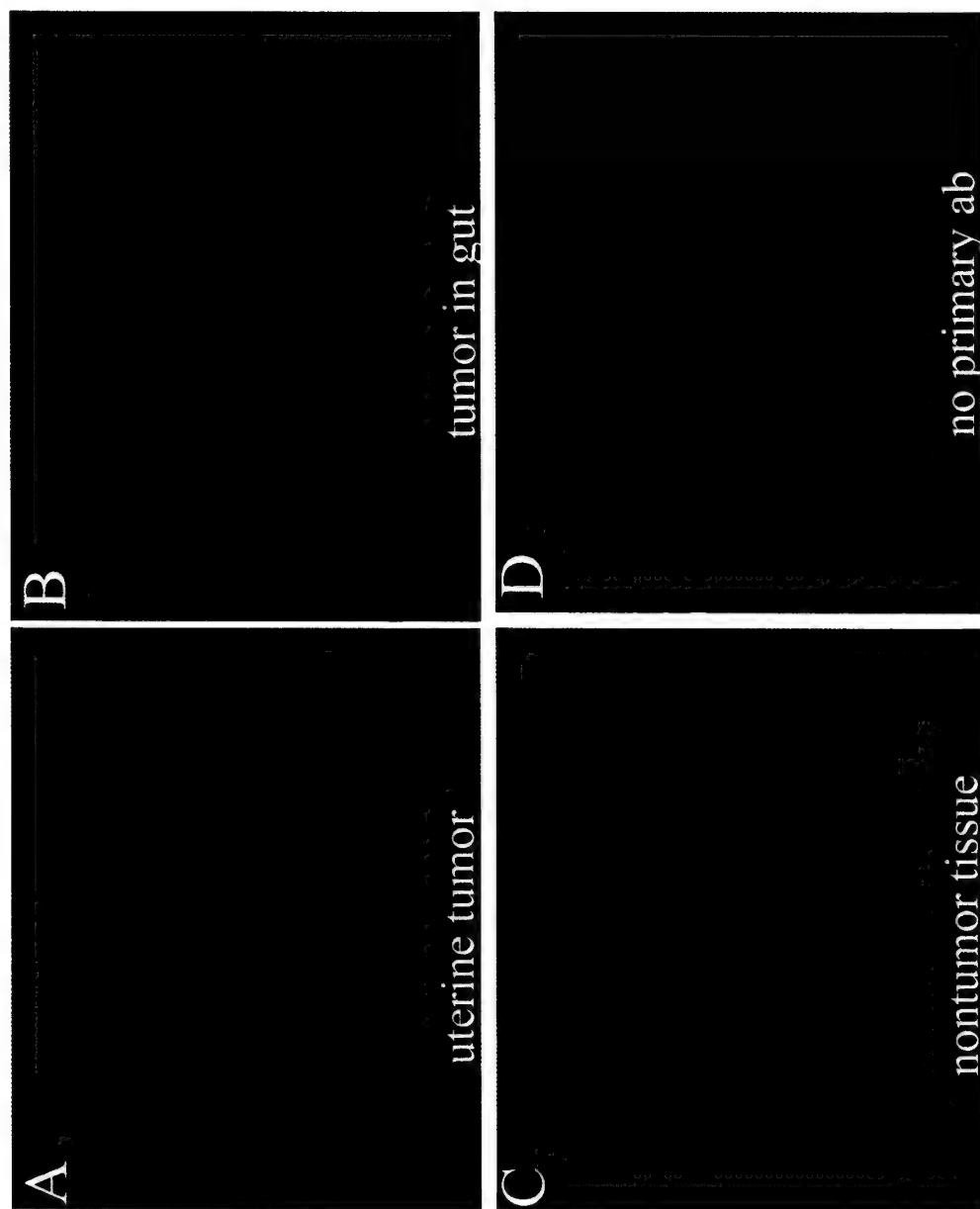


Figure 9. Detection of VSV-G marker for mutated NF2 gene in schwannomas from NF2 transgenic mice. Paraffin sections from the tumors illustrated in Figure 8, extracted from the mouse shown in Figure 6, were immunostained with a polyclonal antibody to VSV-G. Positive staining was present in sections from the uterine tumor (A, 40X) and the tumor in the gut (B, 40X), but absent in sections from nontumor tissue (C, 40X) and control sections treated with secondary antibody alone (D, 40X).



Figure 10. H and E staining on spinal cord sections from a NF2 transgenic mouse [PO-Sch (39-121)]. Note the schwannoma in the anterior horn of the spinal cord (A, 10X; B, 20X, C, 40X). In this animal, the Schwann cells in the tumor invaded the white matter of the spinal cord and disrupted the normal symmetry of the spinal cord. This tumor (see arrows) was not detected by MRI.



Figure 11. Myelin staining on spinal cord sections from the NF2 transgenic mouse (PO-Sch (39-121) illustrated in Fig.10. Note the lack of staining in the schwannoma in the anterior horn of the spinal cord (A, 5x; B, 20x, C, 40X). This is consistent with evidence suggesting that there is disaggregation of myelin sheaths in small vestibular human schwannomas (Sans et al., 1996)

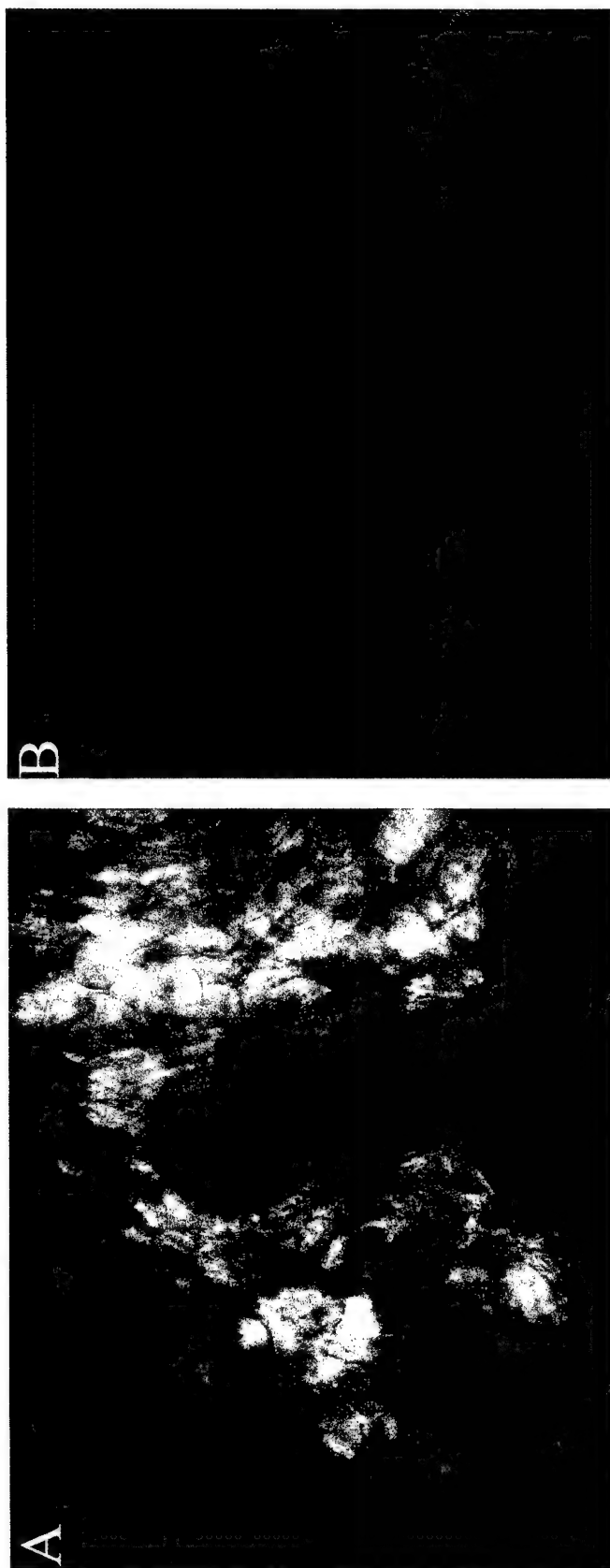
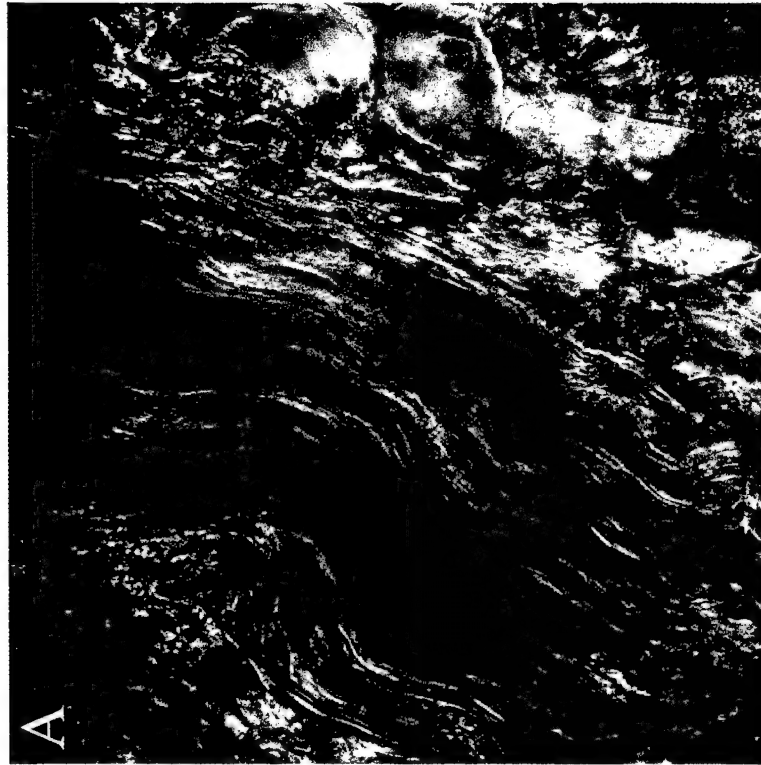
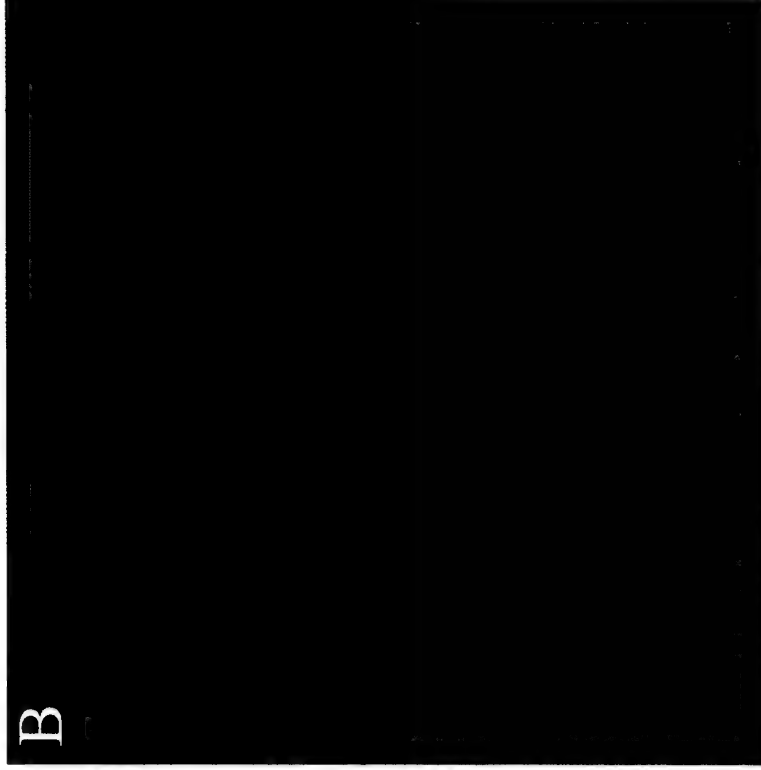


Figure 12.  $\beta$ -galactosidase expression in tumor sections which were injected with hrR3 ( $2.3 \times 10^8$  PFU) . 3 days after injection of virus into the tumor, tumor tissue was removed from the animal, fixed for 24 hrs in 4 % paraformaldehyde, then placed in 25 % sucrose for several days. Tissue was frozen, cut into  $40 \mu\text{m}$  sections, and stained with X-gal buffer [ $1 \text{ mg/ml}$  5-bromo-4-chloro-3-indoxyl  $\beta$ -galactosidase,  $5 \text{ mM K}_3\text{Fe}(\text{CN})_6$ ,  $5 \text{ mM K}_3\text{Fe}(\text{CN})_6$ ,  $-3\text{H}_2\text{O}$ ,  $2 \text{ mM MgCl}_2$  in  $0.1 \text{ M}$  sodium phosphate buffer ( $\text{pH } 7.4$ )] at  $37^\circ\text{C}$  for 12h.  $\beta$ -galactosidase expression is present in tumor tissue infected with hrR3 (A, 40X), indicating infection with HSV vectors, but absent in tumor tissue which was not injected with hrR3 (B, 40X).



X-gal staining



S-100 staining

Figure 13.  $\beta$ -galactosidase expression and S-100 staining share a similar pattern of distribution in tumor tissue infected with hrR3. Tumor tissue was infected with hrR3 ( $2.3 \times 10^8$  PFU) and 3 days later removed from the animal, fixed for 24 hrs. in 4 % paraformaldehyde, then placed in 2.5 % sucrose for several days. Tissue was frozen, cut into 40 micron sections, and stained with X-gal buffer. Sections from the same tumor illustrated in Fig.12.  $\beta$ -galactosidase was present in tumor tissue injected with hrR3 (A, 100X) and shared a similar pattern of distribution with S-100 staining (B, 100X), suggesting that the schwannomas themselves are infectable with hrR3.

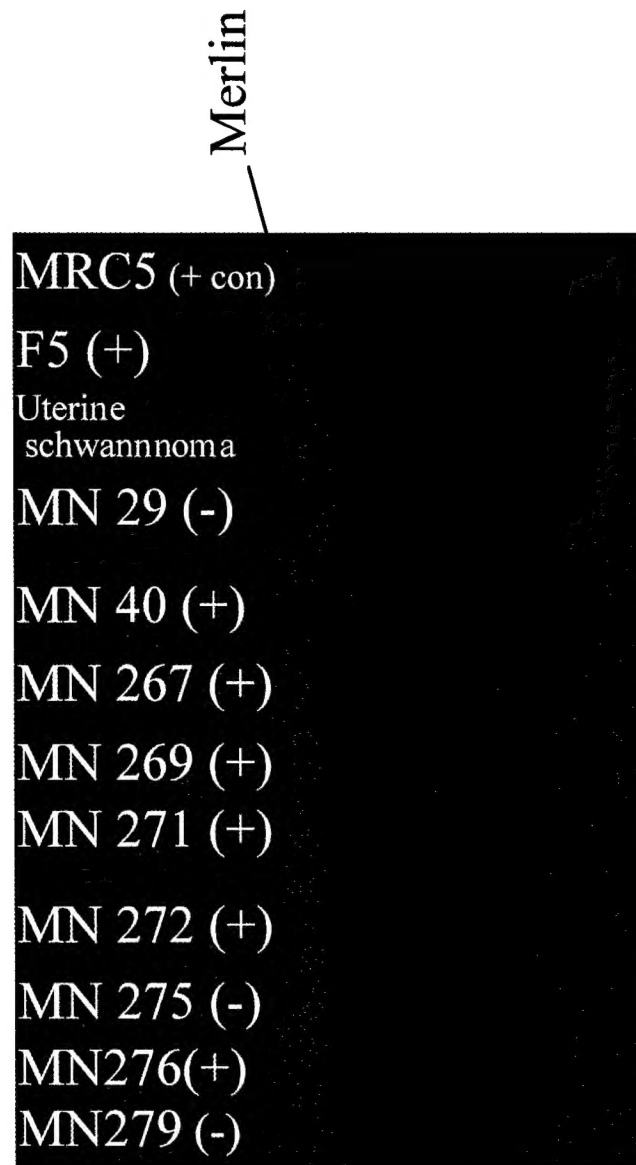


Figure 14. Western blot of cell homogenates from 10 different meningioma lines derived from human patients. One hundred micrograms of protein were loaded in each lane of a 10 % SDS gel. Ponceaus S staining was done to verify that equal amounts of protein were loaded in each lane. MRC5 is a human fetal fibroblast cell lysate which serves as positive control for merlin. A band about 66 kD represents merlin, the protein product for the NF2 tumor suppressor gene. Primary antibody used was 1:30 dilution of MP8 (developed in Dr. V. Ramesh 's laboratory) and the secondary antibody was a 1:10,000 dilution of Goat anti-rabbit-IgG-HRP. Film exposure time was 1hr.

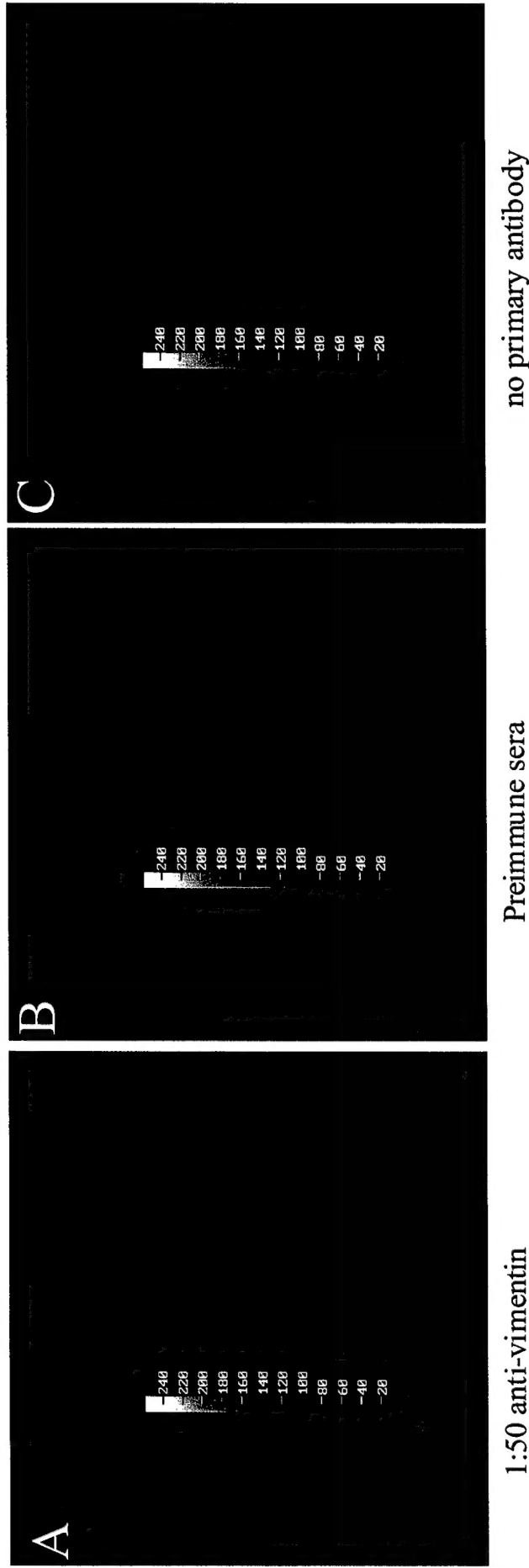


Figure 15. The malignant meningioma cell line F5 grown in cell culture stains positively for vimentin (A, 40X), which is a marker for meningioma cells using a monoclonal antibody to vimentin at a dilution 1:50. Panels B and C are controls, showing that there was no immunoreactivity with preimmune sera alone (B, 40X) or with only secondary antibody and no primary antibody (C, 40X), suggesting that the immunofluorescence in (A) is specific to the presence of vimentin in the F5 cells.



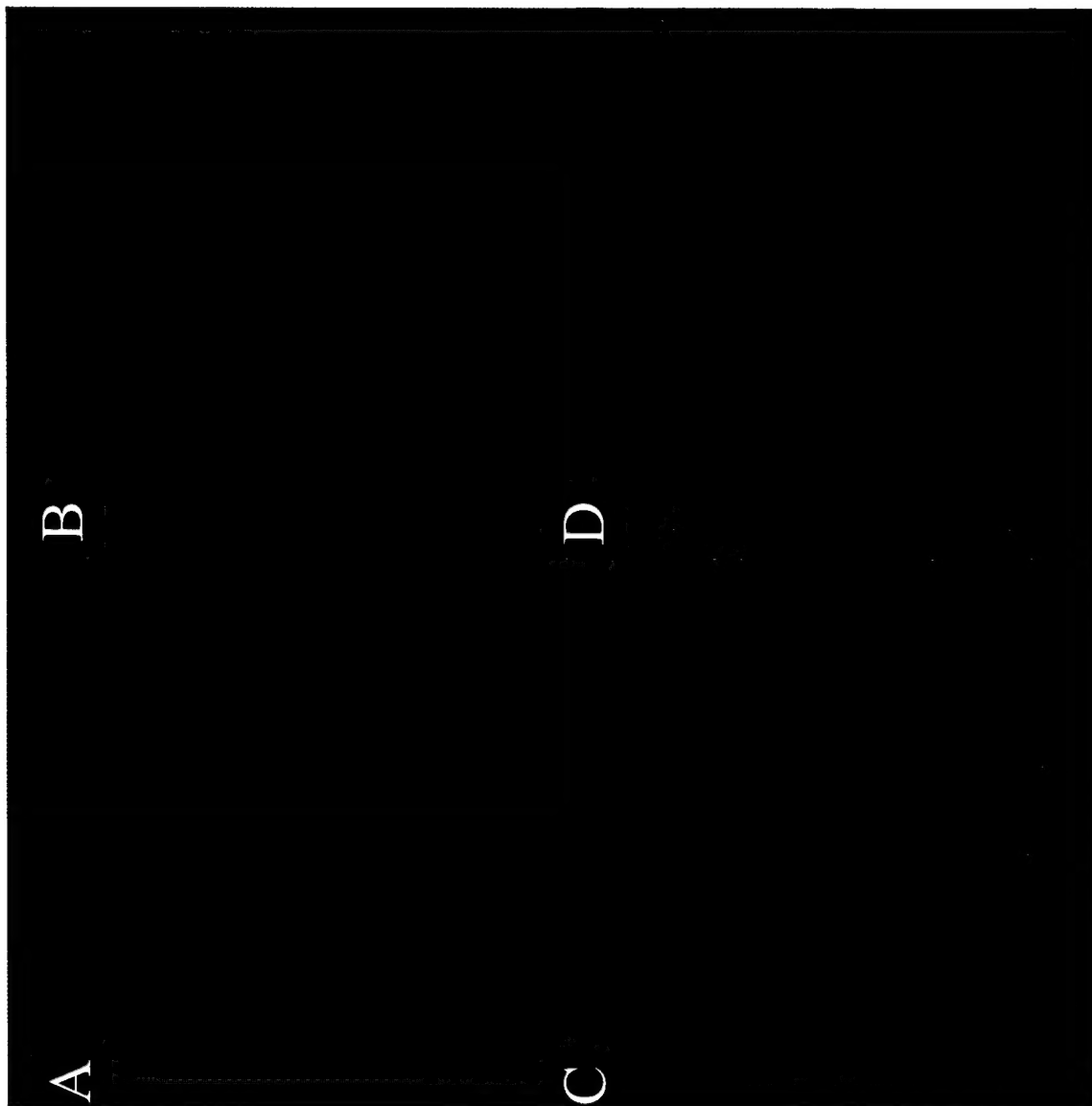
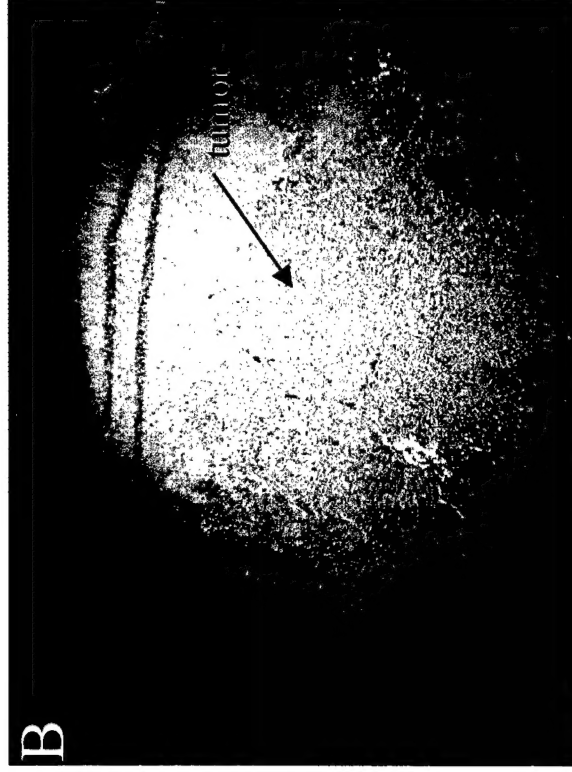


Figure 16. The malignant human meningeoma cell line F5 is infectable in cell culture with a CMV-driven HSV amplicon containing EGFP. Cells were infected at a MOI = 0.5 (B, 20x), MOI = 1 (C, 20x), and MOI=2 (D, 20X) for 48 hrs. prior to be fixed with 4 % paraformaldehyde and examined for GFP fluorescence. Approximately 30 % of cells were infected at a MOI =2.



Control injection



F5 injection

Figure 17. Formation of tumor following injection of the malignant meningioma cell line F5 into right ventricle of nude mouse.  $5 \times 10^8$  F5 cells suspended in DMEM w/ 10 % FBS (experimental) or DMEM w/ 10 % FBS (control) were injected into the right ventricle of nude mice. 4 weeks after injection, mice were perfused with 4 % paraformaldehyde, their brains removed, and paraffin sectioned. H and E staining shows that injection of F5 cells into the right ventricle of a nude mouse resulted a tumor in the ventricle and surrounding parenchyma (B, 50x), while no tumor was present in control injection (A, 50x).